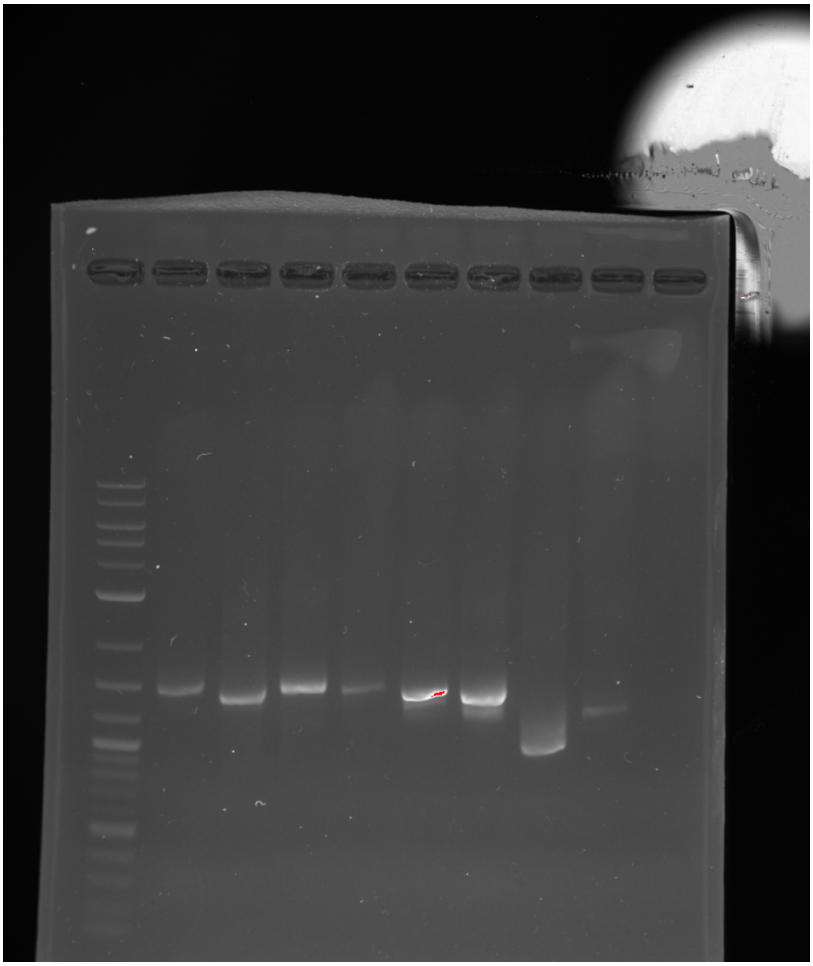
Week 7

July 18, 2016

What We Did Today:

PCR inserts for Gibson assembly (all ADARs, APOBECs, and dCas9)
Ran PCR products on gel
Made liquid cultures of BE2 (3 cultures)

Gel 1

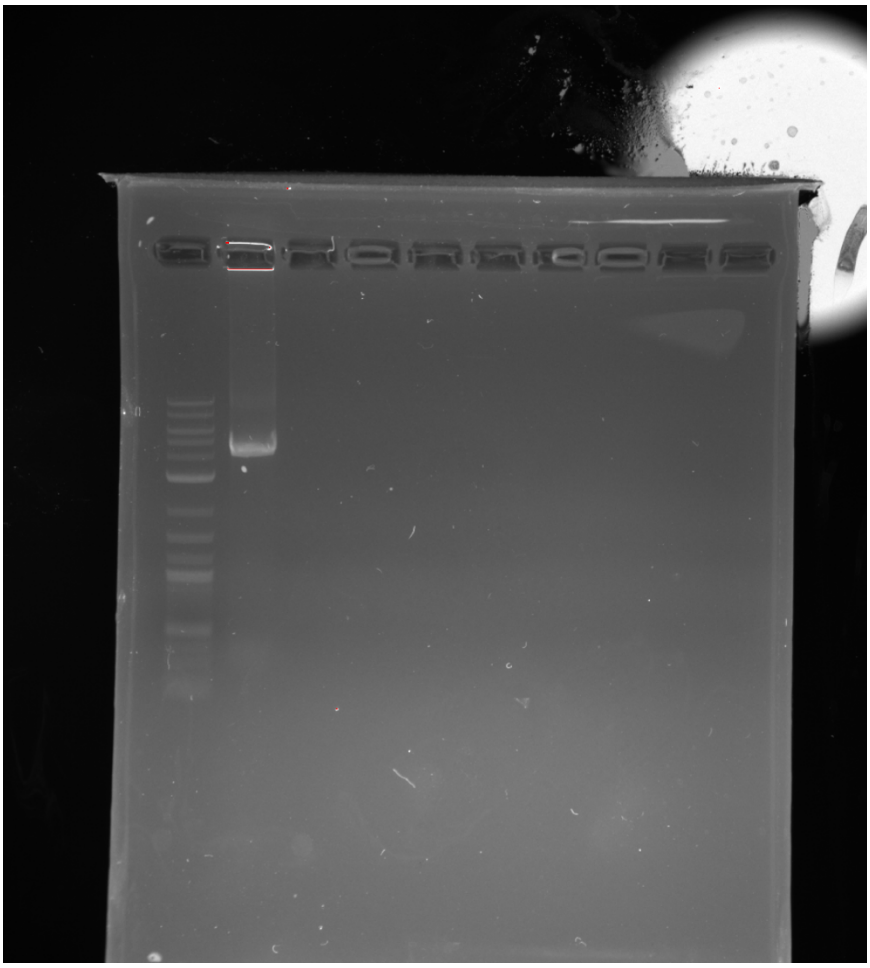


Gel 1: ADAR and APOBEC Inserts
Lane 1 – Ladder
Lane 2 – ADAR 1-1x
Lane 3 – ADAR 1-2x
Lane 4 – ADAR 1-3x
Lane 5 – ADAR 2-1x
Lane 6 – ADAR 2-2x
Lane 7 – ADAR 2-3x
Lane 8 – APOBEC 1x
Lane 9 – APOBEC 2x
Lane 10 – APOBEC 3x

Analysis:

The ADARs are all around the right size for the insert, however, the linker lengths do not look correct. This may be because we may have mixed up the linker lengths. The APOBEC 1x is correct, however we are unsure of what happened to APOBEC 2x and 3x. We will run again tomorrow on a gel infused in SYBR Green to check if the issue is with the binding of the SYBR with the samples.

Gel 2



Gel 2: dCas9 Insert
Lane 1 – Ladder
Lane 2 – dCas9 Insert

Analysis:
The dCas ran right where it should have, showing that the insert is the correct size with primers. It is ready for Gibson assembly.

PCR for restriction digest
Tube:
1 – ADAR1-1x
2- ADAR1-2x
3- ADAR1-3x
4- ADAR2-1x
5- ADAR2-2x
6- ADAR2-3x
7-APOBEC-1x
8- APOBEC-2x
9- APOBEC-3x
PCR:
22uL water

1uL template
1uL each primer
25uL mastermix

Tested Ligating DNA at different times:
5min, 15 min, 30 min, 60 min, at room temp
One incubated at 16 C overnight
-2 uL vector+15 uL water+2 uL buffer+1uL ligase
Control: 2 uL vector + 16 uL water

- PCR
  ADAR1 1X-3X, ADAR2 1X-3X, APOBEC 1X-3X, dCas9
- Gel
  - 5 µL PCR product
  - 1 µL dye

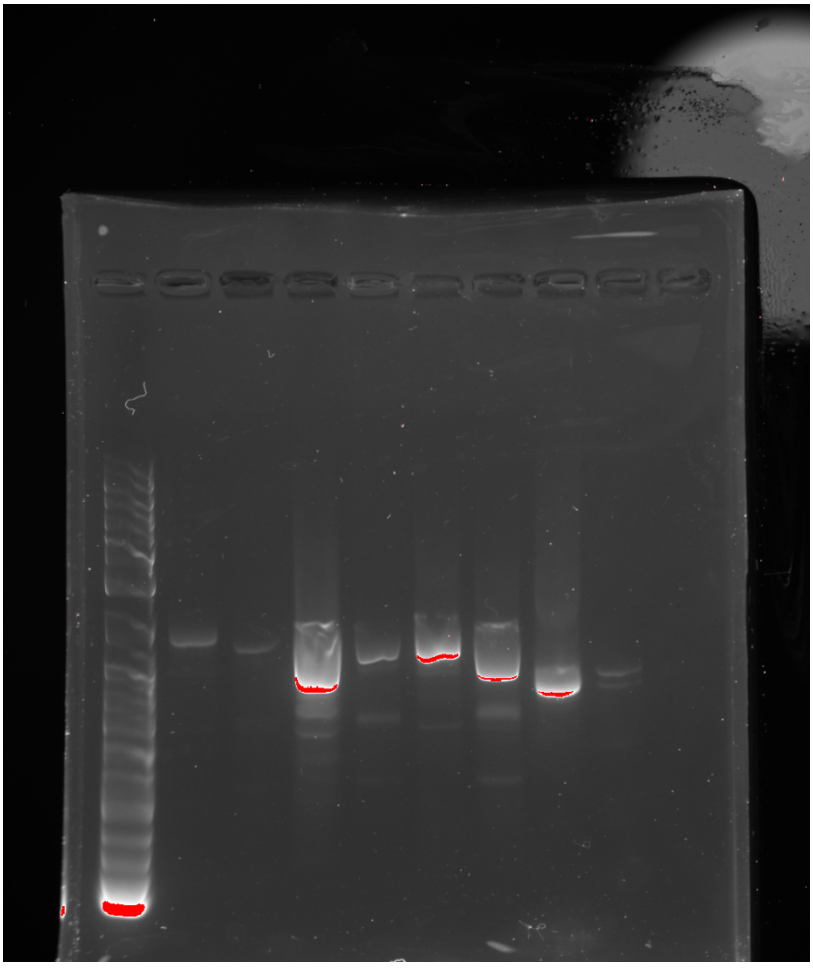
  (ADARs/APOBEC and dCas9 run separately)
- PCR clean up for all

July 19, 2016

I swear to God, I will eat this plate if there are no clones here.
-Natalie 2016

What We Did Today:
Ran ADAR and APOBEC inserts on SYBR Green infused gel to check for Gibson assembly (6ul SYBR was added to agarose and TAE before pouring into gel box)
Mini-prep BE2 samples (made glycerol samples)
Made gels using water for Women in Science Camp tomorrow

Gel 1



Gel 1: ADAR/APOBEC in SYBR Infused Gel
Lane 1 – Ladder
Lane 2 – ADAR 1-1x
Lane 3 – ADAR 2-1x
Lane 4 – ADAR 1-2x
Lane 5 – ADAR 2-2x
Lane 6 – ADAR 1-3x
Lane 7 – ADAR 2-3x
Lane 8 – APOBEC 1x
Lane 9 – APOBEC 2x
Lane 10 APOBEC 3x

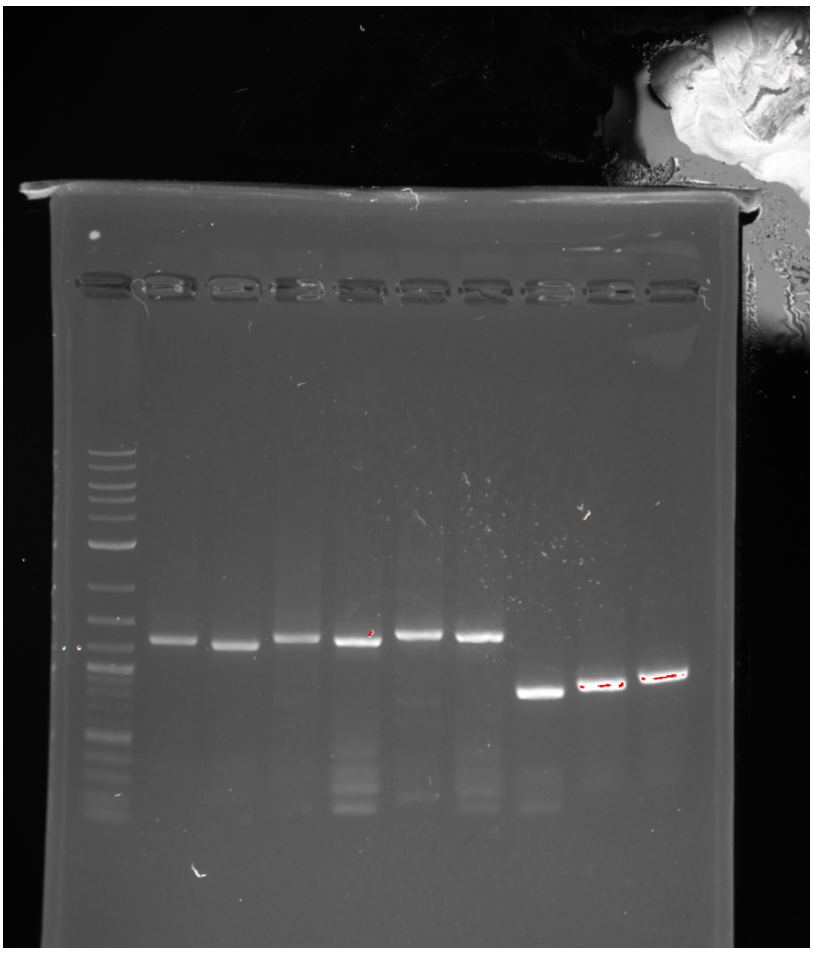
Gel 1: ADAR/APOBEC in SYBR Infused Gel

Analysis:
The ADARs once again ran around the correct place, however the linker lengths did not run where they should have. The APOBECS also ran weird with the 3x linker APOBEC not showing up at all. We discarded these inserts and redid the PCR.

PCR Mixture

1.) Primers were diluted 1:10 with water (9ul water, 1ul Primer)
2.) Components were added to PCR tubes
    1.) 22ul Water
    2.) 1ul Template (ADAR 1, ADAR 2, and APOBEC with all extensions)
    3.) 1ul Primer 1 (3' XTen)
    4.) 1ul Primer 2 (5' dCas)
    5.) 25 ul MasterMix
3.) Tubes were run in the PCR Machine under the iGEM Colony Protocol for 50ul tubes
4.) 5ul of each sample was run on a gel

Gel 2



Gel 2: ADAR and APOBEC Inserts Attempt 2
Lane 1 – Ladder
Lane 2 – ADAR 1-1x
Lane 3 – ADAR 2-1x
Lane 4 – ADAR 1-2x
Lane 5 – ADAR 2-2x
Lane 6 – ADAR 1-3x
Lane 7 – ADAR 2-3x
Lane 8 – APOBEC 1x
Lane 9 – APOBEC 2x
Lane 10 – APOBEC 3x

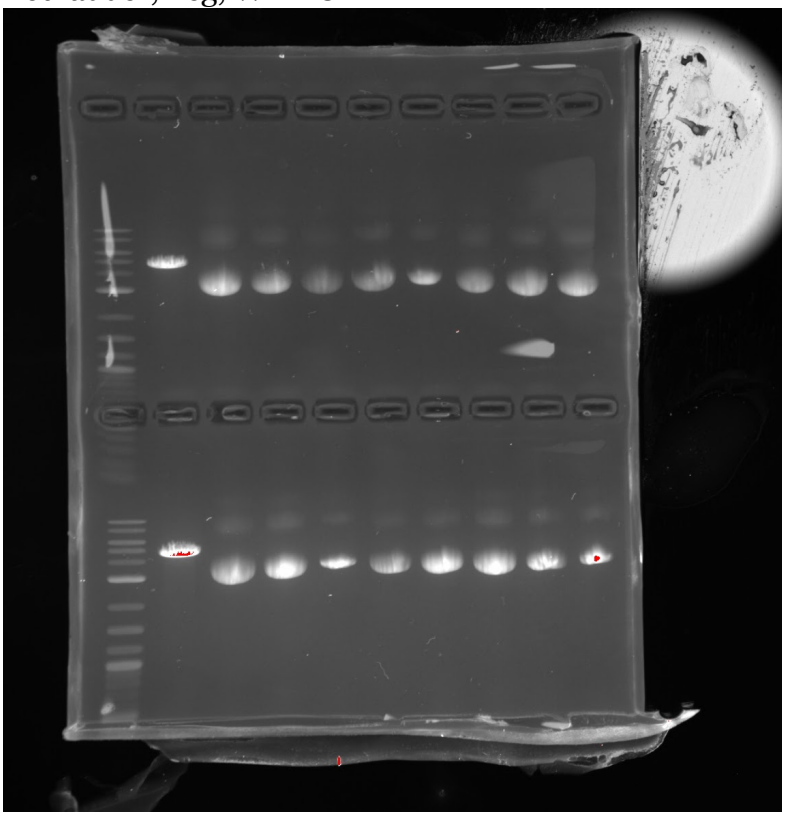
Analysis:
ADAR and APOBEC are in the correct spots. The linkers increase the way they should! We are ready to try Gibson Assembly!!!!!!

0.6g agarose 60mL 1xTAE 6uL SYBR to make SYBR in the gel

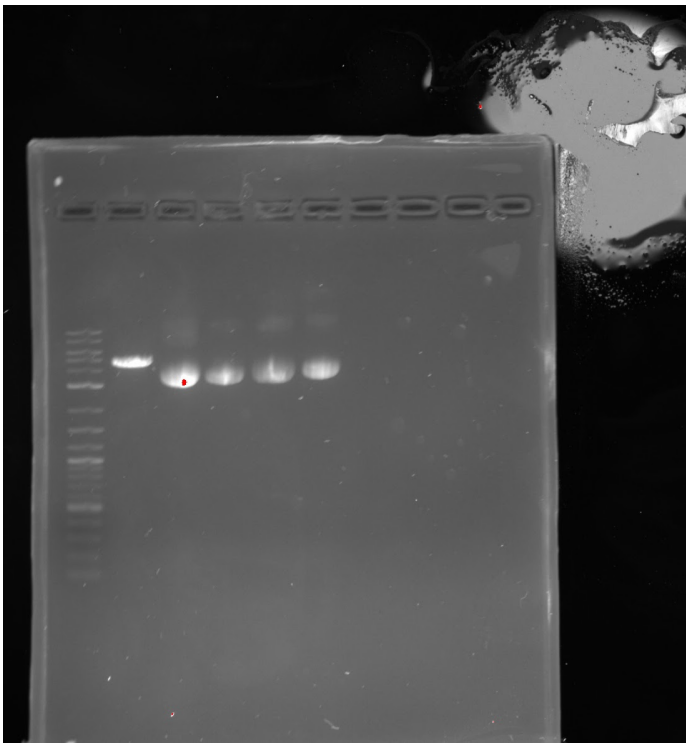
Miniprepped PTC and WT betaglobin run in gel w/ empty GFP
If it looks good digest with Xho1 and BamHI and run again

Colony PCR:
Colonies in 25uL water, boil 95 degrees 10 mins
Vortex
7.5 colony
7.5 2x PCR mix
.1 primer 66 (5' Globin)
.1 Primer 67 (3' Globin)
Make a master mix of above (for 25)
PCR machine:
94° 2 min
94°30 sec-
55°45 sec-
72° 60 sec-
Repeat the above 3 for 35 cycles
72° 2 min

Gel 1 (double)
Top ladder, neg, PTC 1-8
Bot ladder, neg, WT 1-8



Gel 2 ladder, neg, PTC 9,10 WT 9,10



Digest inserts (ADAR1, ADAR2, APOBEC) overnight with NcoI and BglII

Transformed Ligation test samples on 2x AMP
Made primers for creating biobrick vectors

- Miniprep
  - β-globin clones
- Primer design for BioBricks
  - GFP/ β-globin

July 20, 2016

Ligations (25)
Cut vector and insert w/ enzymes 2hr, SAP vector, heat kill both 20 mins

Ligation mixture mix 5 mins then immediately transform, 0, 2uL and 5uL of insert
Amp2x for insert, Kan for PTC/WT

Miniprepped ATG/ACG eGFP DNA
Test digested with BamHI+XbaI

- Salt buffer gels
- Women & Science activities

July 21, 2016

What We Did Today:

Gibson Assembly of APOBEC 1x, 2x, and 3x with dCas
Transformed Gibson Assemblies into DH5(alpha) cells
Made Competent DH5(alpha) cells
Transformed new DH5(alpha) cells using Cell Competency Test Kit
Made LB Liquid
Autoclaved Water

Gibson Assembly Protocol

1.) Set up reaction on ice
- -100 ng vector -> pRETRO-ON (cut with Not1 and BamH1)
- -150 ng dCas Insert
- -200 ng APOBEC Insert
- -10 ul Mastermix
- -Bring up to 20ul Total Volume with Water

2.) Place in PCR machine set at 50 degrees Celsius infinity for 30 minutes
3.) Store at –20 degrees Celsius until ready for Gibson transformation protocol

APOBEC 1x Reaction Mixture

1.23 ul pRETRO-ON
1.90 ul dCas9 Insert
4.18 ul APOBEC 1x Insert
10 ul Mastermix
2.69 ul Water

APOBEC 2x Reaction Mixture

1.23 ul pRETRO-ON
1.90 ul dCas Insert
4.26 ul APOBEC 2x Insert
10 ul Mastermix
2.54 ul Water

APOBEC 3x Reaction Mixture

1.23 ul pRETRO-ON
1.90 ul dCas Insert
4.19 ul APOBEC 3x Insert
10 ul Mastermix
2.68 ul Water

Negative Control

1.23 ul pRETRO-ON
10 ul Mastermix
8.77 ul Water

Gibson Assembly Transformation Protocol

1.) Thaw competent cells on ice
2.) Add 2ul of chilled assembly product to 50 ul DH5(alpha) cells
3.) Place mixture on ice for 30 mins
4.) Heat shock for 30 seconds at 42 degrees Celsius
5.) Transfer tubes to ice for 2 mins
6.) Add 950 ul of room-temperature SOC to tube
7.) Incubate at 37 degrees Celsius for 60 minutes and shake
8.) Warm selection plates to 37 degrees Celsius
9.) Spread 100 ul of cells onto selection plates (using AMP for negative control)
10.) Incubate overnight at 37 degrees Celsius

About 7 colonies grew from the insert
A lot on neg control for globin

Used instant ligation, 5uL instant ligation pipet up and down 10 times and transform immediately

Took ligation mix from the day before for the insert and transformed with 10uL of mix. Added SOC instead of LB in transformation

Thawed HEK293T cells

Transformed samples of positive ATG/ACG eGFP clones to make glycerol stocks

• LB agar plates (2X Amp, 1X Cam)

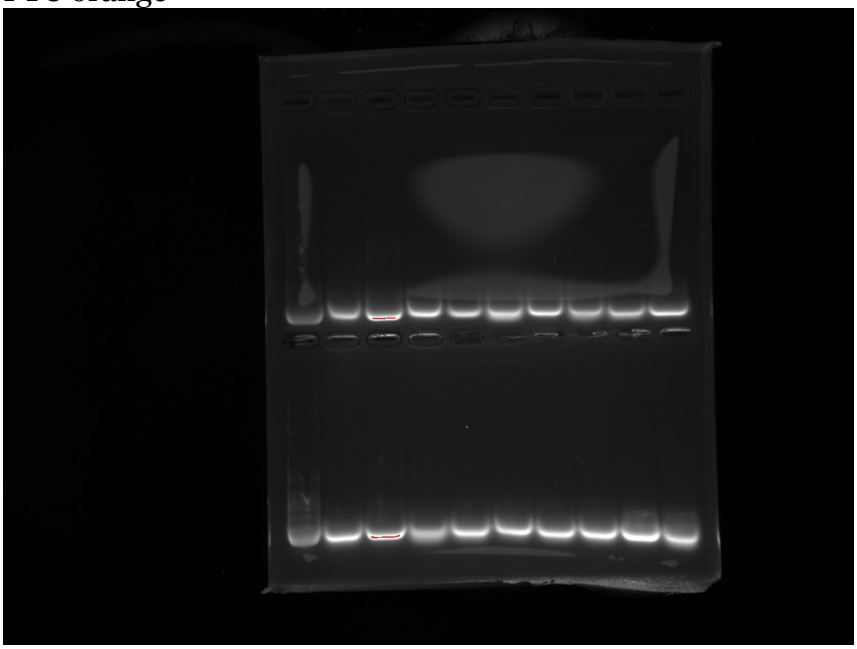
July 22, 2016

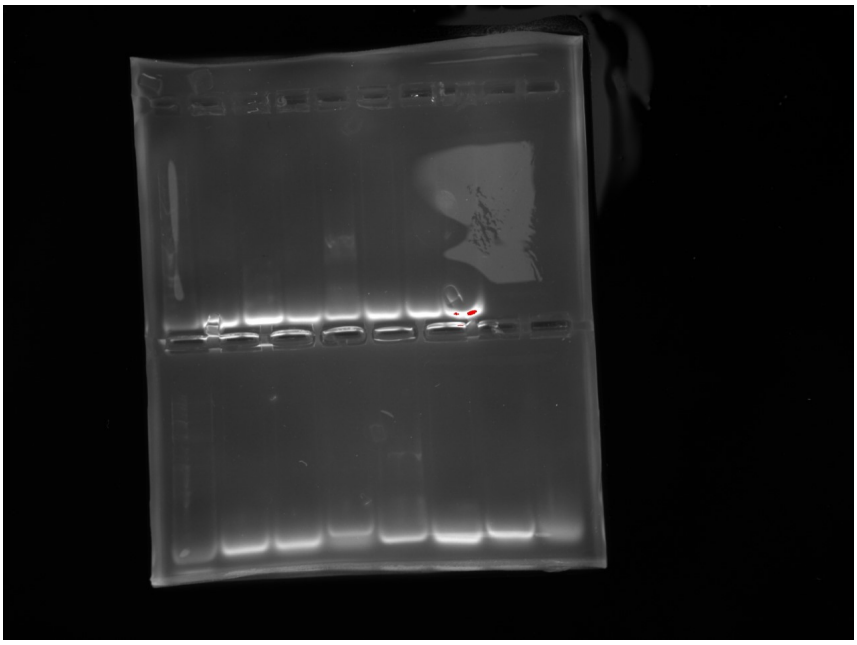
What We Did Today:

Retransformed Gibson Products using 10 ul of Product with 50 ul cells

Plated 3 of each reaction

Colony PCR
Color Key:
WT green
neg control black
pos control red
PTC orange





HEK293T cell maintenance

• Glycerol stocks
• Miniprep
    dCas9-ADAR ligations (7 samples)
    - Concentration: ~160-400 ng/µL
• Digest
    Enzyme: BglII
    - 0.5 µL enzyme
    - 2.5 µL DNA (dCas9-ADAR)
    - 1 µL buffer (3.1)

(10 µL total)